NITROGEN FIXATION IN LAKES: RESPONSE TO MICRONUTRIENTS AND EXPLORATION OF A NOVEL METHOD OF MEASUREMENT

A thesis submitted
To Kent State University in partial
Fulfillment of the requirements for the
Degree of Master of Science

By

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May, 2018

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II. ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Darren Bade, for his investment in the completion of my research. I am truly grateful for his mentorship, his patience, his support, and the knowledge that he has passed down to me. Thanks to Dr. Mark Kershner and Dr. David Costello for serving as committee members for this thesis and for providing additional support and knowledge that was crucial to its completion.

I would also like to extend my thanks to Bree Richardson and Dr. Lauren Kinsmen-Costello for their time and equipment that was instrumental in the analysis of many of my water samples. Additionally, thank you to Dr. Joseph Ortiz and Bowling Green University for their collaboration and their aid in sample collection and field measurements for Lake Erie. Thank you to Bill Zawiski, Greg Orr, and Scott Winkler with the Ohio Environmental Protection Agency for their knowledge and advice on field sites and collection protocols.

I would also like to give a special thank you to Christopher Boehler, Cynthia Perkovich, Lauren Baldarelli, and Bree Richardson for critical help both in the field and in the lab. They were very generous and knowledgeable, which made conducting my research a much smoother process and overall more enjoyable.

Lastly, I would like to thank my family for their continued love and support. I would not be the person I am now without them.

Bethany Schmidt

May 1, 2018, Kent, Ohio
CHAPTER 1

Algal management in freshwater systems and the complication of nitrogen fixation

1.1 An introduction to algal blooms

Phytoplankton are a natural and important component of aquatic ecosystems, but over the last few decades there has been an increase in the number of incidents of problematic, high-density bloom events (Wells et al. 2015). The term phytoplankton includes a large diverse group of microscopic organisms such as dinoflagellates, diatoms, green algae, and cyanobacteria. These organisms are photosynthetic, meaning they use energy from the sun to produce food required for growth and development. Sunlight has limited penetration through a water column, the depth of which is dependent on the level of turbidity or the number of suspended particles in the water (Kirk 1985). Since light is a key requirement for survival, most phytoplankton are located near the surface of the water column to remain in the limited photic region. As primary producers, phytoplankton occupy the base of aquatic food webs, serving as a crucial food source for zooplankton and small fish (Lindsey and Scott 2010). However, when phytoplankton undergo rapid proliferation that surpasses grazing rates, phytoplankton communities reach high densities that result in a layer of scum on the surface of the water is termed an algal bloom.

The presence of algal blooms in freshwater ecosystems can have large negative impacts on water quality, aquatic and terrestrial organisms, public health, and the economy (Anderson et al. 2000). Freshwater systems serve many ecological and economical functions such as activities tied to tourism and recreation, breeding grounds and habitat for aquatic life, nutrient
transportation to downstream systems, irrigation for agricultural lands, a source of drinking water, and management of waste water. (Wetzel 2001). Any biological or chemical variable that impacts water quality can therefore have large negative effects on both the health of the environment and the human population.

As early as the 1940’s, algal blooms started gaining attention with initial complaints surrounding the smelly and repugnant layers of scum that effected recreational activities and sales from tourism (Hasler 1947). Areas of tourism that often experience losses in sales are those affiliated with travel expenses and lodging, restaurant and gift shop sales, fishing equipment sales, and boat rentals. In Ohio alone, the decrease in tourism and recreation activities caused by water pollutants and algal blooms was estimated to be over $40 million over a 2-year period (US EPA 2015). However, the negative impact of algal blooms extends far beyond aesthetics and declines in tourism and recreation.

In addition to the distasteful appearance of algal blooms, over 40 different genera of cyanobacteria can produce toxins that cause acute and chronic illnesses in humans and animals. The symptoms of these toxins can include respiratory and digestive issues, abnormal body growths, loss of memory, seizures, and even death (Sellner et al 2003). When an algal bloom becomes dominated by a toxic or otherwise harmful species of phytoplankton it is termed a harmful algal bloom (HAB). Examples of harmful algal blooms here in Ohio are blooms dominated by cyanobacteria that produce microcystin toxins such as *Mycrocystis, Planktothrix, Anabaena, Anabaenopsis*, or *Nostoc* (Sivonen 2009). High toxin concentrations in freshwater ecosystems has been linked to increased mortality rates of aquatic invertebrates, fish, water fowl, and even small mammals (Ibelings and Havens 2008 and Sellner et al. 2003). Contaminated water supplies have led to major health and economic issues, such was the case with the Toledo
Water Crisis in August of 2014. In this situation, Toledo’s drinking water supply was contaminated with microcystin, which resulted in a state of emergency a “do not drink” order lasting almost 3 days (Jetoo et al. 2015). In Ohio, the city of Celina spent over $13 million over a 2-year period for water treatment of a single contaminated water supply, Grand Lake St. Marys (US EPA 2015). The overall health of freshwater ecosystems can therefore play a crucial role in the economic success and health of local communities.

Non-toxic algal blooms can also be harmful to aquatic ecosystems, impacting multiple ecosystem processes. Some micro and macroalgae have physical characteristics such as spine-like structures that can become lodged in the gill slits of fish, causing damage and even death (Glibert and Anderson 2005). Less palatable species of phytoplankton, both toxic and non-toxic, are a less desirable food source for zooplankton and small fish. Selective grazing of herbivores on competing species of phytoplankton can facilitate the growth and overall dominance of the toxic or less palatable species of phytoplankton in freshwaters (Buskey 2008). Once the phytoplankton community becomes dominated by less palatable species, the rates of grazing can decrease, causing a decrease in the transfer rate of energy and nutrients required for growth to upper trophic levels (Sunda et al. 2006). Furthermore, the large amount of organic material remaining after the death of the phytoplankton can cause an increase in the rates of decomposition, stripping the water of oxygen (Zhou et al. 2015). The resulting hypoxic zones can lead to extensive fish kills over a very short period, causing a loss in revenue and food associated with recreational and commercial fishing. Along with the multitude of negative effects observed, algal blooms have correlated with decreases in overall biodiversity and changes in the structure of food webs (Jones and Brett 2014).
**1.2 Eutrophication and the importance of nutrients**

One of the main causes of algal blooms is increases in anthropogenic nutrient enrichment that’s leads to eutrophication and rapid growth of phytoplankton (Heisler et al. 2008). Eutrophication is a term that has been used since 1907 to describe the situation in which there is an excessive input of key limiting nutrients required for growth and development into a body of water, leading to the prolific growth of plant and algal communities (Hutchinson 1973). The human population has impacted water quality of freshwater systems and in turn the ecological and economical functions of these ecosystems. As early as the 1940’s, ecologists began to suspect that the overuse of fertilizer application in large scale agricultural fields and residential gardens and the subsequent runoff of excess nutrients into aquatic systems causes the proliferation and spread of algal communities (Hasler 1947). Metropolitan areas are expanding in both area and population density, and with this expansion there has been a corresponding increase in anthropogenic activities tied to nutrient enrichment. Activities such as the use of nitrogen and phosphorus-based fertilizers and detergents, growing livestock, the burning of fossil fuels, mining of phosphorus rock, and industrial activities have increased nitrogen and phosphorus inputs to terrestrial and aquatic systems (Mackenzie et al. 2002, USGS 2008). The global cycling of macronutrients such as nitrogen and phosphorus have been heavily altered by anthropogenic activities, with an increase in mobilization of nutrients that has been linked to changes in ecosystem function and diversity (Falkowski et al. 2000, Galloway et al. 2014, Smith et al. 2002). Changes to the concentration of key biological nutrients in freshwater systems can have major consequences such as eutrophication.

Nutrient over-enrichment and eutrophication have impacted numerous lakes located across northern Ohio and is considered a main cause of frequent harmful algal blooms observed
in Lake Erie (Cooke et al. 1977, Olive and Higgins 1981, Stow et al. 2015, Youger 1982). A common belief early on was that large lakes were able to dilute the excess nutrients more successfully than smaller lakes, preventing the formation of eutrophic conditions and algal blooms (Davis 1964). More current information has offered support against this belief, as several of the large Great Lakes have experienced annual eutrophication and algal blooms over the past few years (Wynne et al. 2008, Vanderploeg et al. 2001). Lake Erie, for example, has experienced an increase in the abundance of harmful algal blooms and other phytoplankton communities over the past several years, and the composition of the phytoplankton community has changed with toxic species such as Mycrocystis and Planktothrix increasing in dominance (Michalak et al. 2013). The linkage between changes in nutrient availability and the development of algal blooms has been supported in multiple classes of aquatic ecosystems, from freshwater lakes and rivers to coastal waters (Anderson et al. 2002).

Ecologists and government officials have recognized the consequences of eutrophication and the importance of algal bloom management, but control of anthropogenic nutrient enrichment has proven difficult. Since the 1940’s, environmental laws have been established in the United States to restrict the amount of water pollution caused by the discharge of chemicals from industries and residential areas with a focus on point-sources (Howarth et al. 2000). Unfortunately, there have been minimal restrictions placed on non-point sources of nitrogen and phosphorus coming into aquatic ecosystems, mostly associated with restricting use of nitrogen and phosphorus-based fertilizers and other compounds. This supply of nonpoint sources of macronutrients such as phosphorus and nitrogen has led to the continual occurrence of harmful algal blooms in Lake Erie and other freshwater bodies.
1.3 Management Techniques: P vs. N Debate

Anthropogenic nutrient enrichment of lake systems has increased the biomass of algae over background levels by 60% globally, resulting in a need to reduce nutrient availability as a bottom-up control mechanism to reduce growth and spread of algal blooms (Lewis et al. 2011). The Law of the Minimum proposed by Justus von Liebig states that processes, whether biological or chemical, that depend on multiple components to operate are overall controlled by the scarcest resource (Gorbon et al. 2011). This concept of a single limiting nutrient on biological processes becomes more complex when examining organisms on the community level. Both macro- and micro- nutrients are required in specific ratios for organisms to maintain their growth and development. The specific ratio of macro- and micro- nutrients required for biological processes and the ability of the organism to uptake and store important nutrients is species-dependent (Klausmeier et al. 2004, Smith 1982, Sterner and Elser 2002). The species-dependent nutrient ratios and species-dependent nutrient immobilization rates of a complex community can lead to situations in which a certain species may be limited by one nutrient while another species is limited by a different nutrient. Furthermore, the availability of multiple nutrients may be near their requirement thresholds such that the addition of one limiting nutrient can cause the community to become limited by another nutrient. Depending on the specific requirements and the concentration of nutrients in the water column, the growth of phytoplankton may therefore be limited either by a single nutrient or multiple nutrients (co-limitation) (Elser et al. 2007). The restriction of these limiting nutrients is how management practices have aimed to reduce algal blooms in freshwater systems.

Phosphorus is known to be an important limiting macronutrient in aquatic ecosystems for phytoplankton production which includes the growth rate and development of algal communities...
Phosphorus is a component of many compounds and structures including genetic material (DNA and RNA), energy transfer compounds (ATP), cell membranes (phospholipid bilayers), and bone/exoskeleton support (apatite) (Butusov and Jernelov 2013). Sources of phosphorus to aquatic systems include weathering of phosphate rock (phosphorite), animal feces, decomposing organic matter, phosphorus-based fertilizers and detergents, and industrial waste water. Management practices to reduce the amount of phosphorus inputs to lakes and streams began in the 1960’s and include injection of aluminum compounds into the water column, the construction of artificial wetlands, the introduction of competing primary producers, reductions in the use of phosphorus-based detergents and fertilizers, manure and livestock management plans, control of industrial and municipal wastewater, and repairing and maintaining healthy riparian and wetland areas (US EPA 1999). In many cases, the reduction of phosphorus inputs to water systems has been successful in reducing the growth of algal blooms in lake systems (Lathrop et al. 1998, Schindler et al. 2008, Schindler et al. 2016). Today, management strategies focus primarily on the regulation of total phosphorus (TP) concentrations in aquatic systems, with a particular interest in soluble reactive phosphorus (SRP) (Carpenter 2008, Cooke et al. 2005, Welch and Lindell 2005). However, in some situations the reduction of phosphorus inputs has had either temporary or unsuccessful results in the reduction of algal communities in lake systems, such as the case with Lake Erie (Michalak et al. 2013). In some instances, whole-lake phosphorus fertilization failed to increase algal biomass, indicating a lack of phosphorus limitation in several Swedish lakes (Holmgren 1984).

One possible explanation why the strategy of reducing phosphorus inputs may be an ineffective management practice for some lakes is that phosphorus inputs can be difficult to control. Environmental laws have been established to restrict the amount of discharge of
macronutrients such as phosphorus from industrial and residential point sources (Howarth et al. 2000). For example, the annual point sources of phosphorus into Lake Erie have declined since 1974, falling from over 8,000 to below 2,000 metric tons per year (Ohio EPA 2013). Ohio laws and regulations of non-point phosphorus sources have only been implemented recently and are very minimal, such as restrictions on manure and phosphorus-based fertilizer application (Staley 2017). In Lake Erie, the annual nonpoint sources of phosphorus entering the lake have not seen a steady reduction like those observed with the point sources since 1974, with the nonpoint sources continuing to range between 3,000 and 12,000 metric tons per year (Ohio EPA 2013). Unless non-point sources can be successfully regulated, the strategy of reducing phosphorus inputs may be unsuccessful at limiting phytoplankton growth in certain lakes.

Another possible explanation why the strategy of reducing phosphorus inputs may be an ineffective management practice for some lakes is the complications of internal phosphorus loading and the storage capacity of phytoplankton. The sediment of lakes and rivers can play a critical role in the cycling of phosphorus. Iron in the sediments of lakes and rivers binds to phosphate ions where they are temporarily stored, allowing the sediments to act as a sink for phosphorus (Chorus and Bartram 1999). Sediments can also release phosphorus into the water column under certain conditions which include high temperature, high decomposition rates, high concentrations of nitrate, and low concentrations of oxygen (Søndergaard et al. 2012). When decomposition of algae decreases the concentration of dissolved oxygen and the water becomes anoxic, the sediments release phosphorus back into the water column acting as a feedback loop to stimulate the growth of additional algal blooms. Similar to the sediments, phytoplankton have the ability to immobilize phosphorus from the water column and store it in their cells until needed (Chorus and Bartram 1999). The storage capacity of phosphorus in phytoplankton can
allow them to continue growth and development even after the availability of phosphorus in the water column has been depleted. When phosphorus inputs are too difficult to control or when there are complications from internal phosphorus cycling, an alternative control measure is needed.

A possible alternative to phosphorus control to reduce the growth of algal blooms is that of nitrogen control. Like phosphorus, nitrogen is a critical macronutrient for organismal growth as it is required to synthesize many biomolecules such as proteins and amino acids, hormone chemicals, nucleic acids found in DNA, and photosynthetic pigments such as chlorophyll (Alberts et al. 1989). Changes to the cycling of nitrogen within a watershed can have major impacts on the availability of nitrogen in aquatic systems and the growth of the phytoplankton community. The availability of nitrogen that can be immobilized by phytoplankton in aquatic ecosystems during the major growing seasons impacts both the density of algal blooms and the maintenance of toxic production (Xu et al. 2009, Scott and McCarthy 2010). Sources of nitrogen to an ecosystem include atmospheric deposition (rain, snow, fog), animal manure, organic matter decay, and the diffusion of dinitrogen gas between the atmosphere and the surface of the water. A sizeable portion of the nitrogen on Earth consists of inert forms of nitrogen that are biologically inaccessible by most biota, including atmospheric dinitrogen gas amounting to 3.7x10^9 Tg and dissolved dinitrogen gas in the oceans and freshwater systems amounting to 1.46x10^6 Tg (Emerson et al. 2002, Sorai et al. 2007, Ward 2012). A portion of this large, inert pool of nitrogen is being converted into biologically available ammonia through the Haber-Bosch process to be used in commercial fertilizers and in the manufacturing of products such as nylon, resins, and plastics (Ghaly and Ramakrishnan 2015). The human population has greatly altered the cycling of nitrogen on Earth through anthropogenic activities such as synthesizing
fertilizers through the Haber-Bosch process, livestock manure, the combustion of fossil fuels, waste water treatment plant outputs, and industrial waste (Ghaly and Ramakrishnan 2015, Vitousek et al. 1997). These anthropogenic activities are estimated to have increased the amount of reactive nitrogen inputs in the United States by 5-fold (Houlton et al. 2013). The natural conversion of this inert pool of nitrogen into reactive nitrogen, however, is through the biological process of nitrogen fixation.

Nitrogen fixation is an anaerobic process that converts inert dinitrogen gas in the atmosphere into biologically available ammonium, which can then be assimilated by biota and incorporated into organic tissue (Figure 1.1). Only organisms that can produce the enzyme nitrogenase have the ability to fix nitrogen, allowing them to continue growth when other reactive forms of nitrogen are diminished. These organisms include bacteria such as rhizobia that form endosymbiotic relationships with Leguminous plants, green sulfur bacteria, methanogens, azotobacteraceae, and certain species of cyanobacteria in aquatic systems (Howarth et al. 1988, Ward 2012).

For cyanobacteria to carry out the process of nitrogen fixation, there must be spatial separation between the oxygen-sensitive nitrogenase enzyme and the site of photosynthesis that produces oxygen as a waste product (Zhang et al. 2006). This spatial separation is achieved by the production of specialized cells called heterocytes whose envelope prevents the diffusion of oxygen into the cell and maintains an anaerobic microenvironment (Flores and Herrero 2010, Golden and Yoon 2003). When a cell undergoes differentiation to form a heterocyte, the protein complex photosystem PSII that normally produces oxygen during photosynthesis is deactivated (Wolk et al. 1994). The envelope of the heterocyte consists of a polysaccharide layer and a glycolipid layer, with the glycolipid layer responsible for reducing the ability of oxygen to
Figure 1.1 The process of nitrogen fixation in cyanobacteria occurs in special differentiated cells called heterocyes, indicated by the red arrow (A). The process requires the enzyme nitrogenase, several co-factors such as iron (Fe) and molybdenum (Mo), and a large amount of energy (ATP) (B).
diffuse into the cell (Zhang et al. 2006). Furthermore, rates of respiration tend to be higher in heterocyte cells compared to normal vegetative cells, which quickly consumes any oxygen that leaks into the cell (Wolk et al. 1994). It is here in the heterocyte that the nitrogenase enzyme is housed. The strong triple bond between the two nitrogen atoms requires a large amount of energy to break, roughly equivalent to 16 ATP, so phytoplankton only produce nitrogenase-containing heterocytes under nitrogen poor conditions (Adams 2000, Berg et al. 2002). The ability to fix nitrogen becomes a competitive advantage under nitrogen-poor conditions, which can promote the growth and dominance of nitrogen fixing species of cyanobacteria. Nitrogen fixation rates have been reported to be relatively low in oligotrophic and mesotrophic lakes (0.1 g N m\(^{-2}\) yr\(^{-1}\)), but eutrophic lakes, such as those impacted by anthropogenic phosphorus enrichment, have yielded much higher rates of nitrogen fixation (0.2-9.2 g N m\(^{-2}\) yr\(^{-1}\)) (Howarth et al. 1988).

Some ecologists have argued that nitrogen control will not suffice as an alternative to phosphorus control due to the complications of biological nitrogen fixation (Schindler et al. 2008, Schindler et al. 2016, Wang and Wang 2009). There is concern that reductions in nitrogen inputs will favor the dominance of nitrogen-fixing cyanobacteria that can compensate for the lack of nitrogen by fixing their own nitrogen using dinitrogen gas. Unlimited biological nitrogen fixation could cause reducing nitrogen inputs to be ineffective in reducing eutrophication. Research conducted at the Canadian Experimental Lake Area has offered support to the concept that nitrogen compensation occurs through nitrogen fixation. This whole-lake experiment is often cited as evidence that nitrogen-fixers can overcome the nitrogen deficiency caused when the lake is enriched with phosphorus (Schindler et al. 2008). Furthermore, management practices for the reduction of phosphorus inputs into lakes and streams is easier and less expensive compared to management practices for the reduction of nitrogen inputs (Wang and Wang 2009).
Some ecologists argue that there is evidence against the concept of phosphorus reductions as the only plausible management practice to reduce algal blooms, and favor instead a joint phosphorus control and nitrogen control management (Elser et al. 2007, Gobler et al. 2016, Lewis et al. 2011, Paerl et al. 2014). Individual and whole-lake experiments have suggested that phytoplankton growth is often limited by both nitrogen and phosphorus, despite the presence of nitrogen-fixing cyanobacteria (Dodds and Smith 2016, Elser et al. 2007, Mostert and Grobbelaar 1987). For example, a whole-lake experiment in the Experimental Lakes Area of Canada showed that nitrogen fixing cyanobacteria were unable to compensate for the lack of bioavailable nitrogen in the long term and the system became nitrogen limited (Scott and McCarthy 2010). Looking at the overall freshwater system, nitrogen fixation by cyanobacteria have been reported to meet less than half of the nitrogen requirements of primary producers when phosphorus is in excess (Howarth et al. 1988, Lewis and Wurtsbaugh 2008, Scott and McCarthy 2010). The inability of nitrogen fixing cyanobacteria to compensate for low levels of biologically available nitrogen suggests that there are environmental or biological factors that limit rates of nitrogen fixation.

1.4 Possible limiting factors on nitrogen fixation

The process of nitrogen fixation relies on a combination of different macro- and micro-nutrients including phosphorus, iron, molybdenum, and boron. Macronutrient limitation of nitrogen fixation is often alleviated by increased runoff and direct inputs from anthropogenic activities. The enrichment of freshwaters with nitrogen and phosphorus is paving the way for micronutrients or other environmental factors to possibly overtake macronutrients as the limiting resource (Downs et al. 2008, Lambert and Davy 2011, Wurtsbaugh and Horne 1983). The
relative availability of these micronutrients has the potential to impact the rate of nitrogen fixation and the overall growth of the cyanobacterial community as one nutrient becomes limiting compared to another.

The nitrogenase enzyme consists of two multi-subunit proteins, Component I and Component II (Mehta et al. 2003, Ward 2012). Component II is the iron protein, or dinitrogenase reductase, which is responsible for transferring electrons to Component I, the molybdenum-iron protein or dinitrogenase. Once the transfer of electrons is complete, the area containing the molybdenum and iron cofactors on Component I serves as the site of nitrogen fixation. The result is the conversion of one mole of dinitrogen gas (N\textsubscript{2}) into 2 moles of ammonium (NH\textsubscript{4}\textsuperscript{+}) (eq. 1):

\[
N_2 + 8H^+ + 16ATP + 6e^- \rightarrow 2NH_4^+ + 16ADP + 16P_i
\] (1)

The ammonium is then converted and incorporated into organic tissue. Some species have an alternative dinitrogenase complex that involves vanadium rather than molybdenum, but the pathway linked to molybdenum is more efficient and preferred by nitrogen fixers (Glass et al. 2012). Genes for the molybdenum-iron protein and the iron protein complexes, the \textit{nifDK} gene and the \textit{nifH} gene respectively, were present in the common ancestor of nitrogen fixers and are highly conserved, which hints at the importance of iron and molybdenum in nitrogen fixation (Raymond et al. 2004).

Due to their role as cofactors in the nitrogenase pathway to breakdown dinitrogen gas, both iron and molybdenum may limit the rate of nitrogen fixation when the macronutrient phosphorus is abundant (Bellenger et al. 2008, Wurtsbaugh and Horne 1983). Iron is the fourth most abundant element that is found in the Earth’s crust (5% iron), and is found in multiple
minerals such as haematite, magnetite, and taconite. Inputs of iron into aquatic systems occurs through the weathering of rocks and soil, however due to its low solubility in water the concentration of iron in freshwater systems is typically below 1 mg iron / L (Molot and Dillon 2003, Shaked et al. 2004). The thresholds of iron concentrations in which phytoplankton growth and development become limited by the availability of iron are species-specific and depend on the availability of other nutrients, but typical iron: phosphorus requirement ratios range from 0.02-0.04 moles (Brand 1991). For example, one ex situ investigation into the effect of iron availability on a specific nitrogen fixer, Anabaena circinalis, revealed an iron concentration threshold for growth at 0.04 mg iron/L (Sun et al. 2005). Iron as a limiting resource for cyanobacterial growth and nitrogen fixation has been well supported (Hyenstrand et al. 2001, North et al. 2007, Orihel et al. 2016). The element molybdenum is rarer than iron, found naturally in the form of molybdenite (molybdenum sulfide) mineral deposits and consisting of less than 0.001% of the Earth’s crust (Canadian Council of Ministers of the Environment 1999). The concentration of molybdenum in rivers and lakes is typically below 20nM (Glass et al. 2013). Just like with iron, the concentration of molybdenum in which phytoplankton growth and development become limited are species-specific and depend on the availability of other nutrients, but typical requirement ratios range from 0.1-3 μmol molybdenum to 1 mol of carbon (Glass et al. 2012). There have been conflicting reports on molybdenum as a limiting resource for nitrogen fixation with some experiments showing molybdenum limitation while others show no response to molybdenum additions (Downs et al. 2008, Glass et al. 2012).

The maintenance and protection of the glycolipid envelope surrounding the heterocyte has been shown to be dependent on the presence of boron (Hyenstrand et al. 2001, Downs et al. 2008). Under boron-deficient conditions, the envelope of the heterocyte undergoes destabilizing
conformational changes which allow for oxygen to diffuse in and deactivate nitrogenase. The result is decrease in both the growth of heterocystic cyanobacteria and the nitrogenase activity level (Bonilla et al. 1990). The element boron also consists of less than 0.001% of the Earth’s crust and it is found in more than 200 boron oxide-containing minerals and compounds such as borax, kernite, colemite, and boric acid (Jansen 2011). Concentrations of boron in United States freshwater systems averages about 0.1 mg boron/L, although this average can reach upwards of 15 mg boron/L in certain regions of the United States (Butterwick et al. 1989). These low concentrations of boron are important to consider, particularly since research has shown that concentrations of boron below 0.1 mg boron/L can limit the growth of phytoplankton (Lewin 1966). Whether directly through the role as a co-factor (e.g. iron and molybdenum) or indirectly through the maintenance of an anoxic environment (boron), micronutrients have the potential to limit the rate of nitrogen fixation when certain macronutrients such as phosphorus are abundant. 

There are other possible environmental and chemical limitations on nitrogen fixation apart from macro- and micro- nutrients. Nitrogen fixation rates and overall cyanobacteria biomass can be significantly impacted by light availability, with heterocystic cyanobacteria favoring high light levels and non-heterocystic cyanobacteria favoring low light levels (Mugidde et al. 2003). The process of nitrogen fixation uses a large amount of energy created from photosynthesis to convert dinitrogen gas into ammonium, which may explain the light constraint on the growth of nitrogen-fixing cyanobacteria (Turpin 1991). Mugidde et al. (2003) also observed increase nitrogen fixation rates and cyanobacterial biomass at lower turbidity levels. Sunlight has a limited ability to penetrate a column of water, and the depth of penetration is dependent on the level of turbidity (Kirk 1985). Although not considered a major issue in freshwater systems, research has also shown a negative relationship between salinity and
nitrogen fixation rates (Herbst 1998). Algal communities and rates of nitrogen fixation can also experience selective grazing of zooplankton which can facilitate the growth and overall dominance of one species of cyanobacteria over another (Buskey 2008). The balance between inter and intra species competition for resources amongst nitrogen-fixing and non-nitrogen fixing cyanobacteria can further influence rates of nitrogen fixation (Vitousek et al. 2002). All these environmental factors, whether chemical, physical, or biological, have the potential to limit nitrogen fixation in freshwater systems.

1.5 Conclusions

Bottom-up control through restrictions of nutrient inputs is necessary to reduce excess volumes of phytoplankton that can cause significant issues for water quality, freshwater food webs, human health, and the economy of local cities (Anderson et al. 2000, Lewis et al. 2011). The debate between phosphorus and nitrogen reductions as efficient management practices for the control of algal blooms is ongoing. However, the idea of a joint phosphorus and nitrogen control management practices is gaining the support of ecologists as research reveals new information about nitrogen fixation’s role in nitrogen cycling (Elser et al. 2007, Gobler et al. 2016, Lewis et al. 2011, Paerl et al. 2014). Research has shown that nitrogen limitation in lakes with nitrogen-fixers is common and reoccurring, suggesting that nitrogen fixation by cyanobacteria is not enough to compensate for the absence of bioavailable nitrogen in the water column to meet their biological requirements (Conley et al. 2009, Scott and McCarthy 2010). A possible explanation for nitrogen limitation in freshwater systems with nitrogen fixing cyanobacteria is environmental or biological factors that is limiting nitrogen fixation. If nitrogen fixation is limited, then nitrogen controls may prove to be an effective alternative to phosphorus
controls. This debate between nitrogen and phosphorus control ties directly into the main goal of this thesis, which is to investigate possible micronutrient limitations on rates of nitrogen fixation.

References


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CHAPTER 2

The effect of micronutrients on nitrogen fixation rates across northern Ohio lakes

2.1 Abstract

Anthropogenic changes in the availability of nutrients in freshwater systems can affect the growth and composition of the cyanobacterial community through changes in biological processes. Limitations on these biological processes are used to develop management plans for reoccurring algal blooms. Nitrogen fixation is a biological process that converts inert dinitrogen gas into biologically available ammonium. In freshwater systems, certain cyanobacteria possess this ability to fix nitrogen, giving them an advantage under nitrogen-poor conditions. Nitrogen fixation and cyanobacterial growth could be limited by phosphorus, but pollution has often created situations where phosphorus is in excess. The process of nitrogen fixation is also dependent on the presence of multiple micronutrients, which are involved either as cofactors or in maintenance of cells. This dependence on micronutrients leads to the question of whether micronutrients could limit nitrogen fixation and cyanobacterial growth when phosphorus is abundant. To investigate the response of nitrogen fixation rates to micronutrient enrichment, I conducted macronutrient (phosphorus) and micronutrient (iron, boron, molybdenum) addition bioassays using water samples collected from 8 field sites in northern Ohio. In 5 of the 8 sites sampled, nitrogen fixation rates were significantly increased by additions of phosphorus. Micronutrient limitation was only observed in 1 of the 8 sites, with the molybdenum addition further increasing rates of nitrogen fixation. If micronutrient limitation on nitrogen fixation rates
is absent or rare in northern Ohio, nitrogen fixation may serve as a hinderance in nitrogen control practices for the reduction of cyanobacteria growth within freshwater systems.

2.2 Introduction

Anthropogenic activities have altered the cycling of nutrients within freshwater systems, and there is now a growing need to monitor and reduce the input of nutrients to manage algal blooms and maintain water quality requirements (Vitousek et al. 1997). The rules of stoichiometry apply to biological processes, meaning organisms require macro- and micro-nutrients in specific ratios to maintain growth and development. The scarcest resource is what ultimately determines the rate of essential biological processes (Klausmeier et al. 2004, Redfield 1958). Anthropogenic changes to the cycling of biologically-required macronutrients such as phosphorus and nitrogen have occurred on a global scale, and the increased mobilization of these nutrients has been linked to changes in ecosystem function and diversity (Falkowski et al. 2000, Galloway et al. 2014, Smith et al. 2002). In freshwater systems, changes in the concentration of nitrogen and phosphorus can have a large impact on water quality and eutrophication, harmful algal blooms, hypoxic zones, and changes in the structure of food webs (Jones and Brett 2014). Management practices have aimed to reduce the input of these macronutrients to create limitations on growth rates of cyanobacteria, but complications have arisen with natural biological processes that can also alter the cycling of nutrients such as nitrogen fixation.

Nitrogen fixation is an anaerobic process that converts inert atmospheric dinitrogen gas into ammonium using the enzyme nitrogenase, and ammonium is then incorporated into organic tissue. The ability to fix nitrogen is limited to specific autotrophic and heterotrophic bacteria including certain genera of cyanobacteria found in freshwater systems (Howarth et al. 1988). The
impact of biological nitrogen fixation on the cycling of nitrogen occurs on both local and global scales, accounting for as much as 110 Tg of N per year entering terrestrial systems or 140 Tg N per year entering the marine systems (Gruber and Galloway 2008). Many ecologists argue that nitrogen control is not an effective management practice to reduce the occurrence and intensity of algal blooms because of this ability of cyanobacteria to fix their own nitrogen when exposed to nitrogen-poor conditions (Schindler et al. 2016). These ecologists believe that the focus of management practices for algal bloom control should therefore focus on phosphorus alone, and it is in phosphorus reductions that management practices today still concentrate on (Carpenter 2008, Schindler et al. 200). However, there is evidence against this idea that phosphorus reductions are the only plausible management practice to reduce algal blooms (Dodds and Smith 2016, Elser et al. 2007, Gobler et al. 2016, Lewis et al. 2011, Paerl et al. 2014). If the process of nitrogen fixation is limited by environmental factors and does not necessarily compensate cyanobacteria for the loss of nitrogen inputs into the watershed, then nitrogen control may prove to be an effective means to manage algal blooms.

The potential for micronutrients to alter the growth and nitrogen fixation capability of cyanobacteria is often overlooked. As a biological process relying on a combination of different macro- and micro- nutrients, nitrogen fixation has the potential to be impacted by the change in nutrient ratios within the water column. Some nutrients that are required for nitrogen fixation include phosphorus, iron, boron, and molybdenum. Iron and molybdenum are important co-factors in the nitrogenase enzyme that reduces atmospheric dinitrogen and therefore have the potential to limit the rate of nitrogen fixation (Bellenger et al. 2008, Wurtsbaugh and Horne 1983). Another major requirement for nitrogen fixation in cyanobacteria is the spatial separation between the oxygen-sensitive nitrogenase enzyme and the site of photosynthesis that produces
oxygen as a waste product (Zhang et al. 2006). Certain cyanobacteria house nitrogenase inside specialized heterocyte cells whose envelope prevents the diffusion of oxygen into the cell (Flores and Herrero 2010, Golden and Yoon 2003). The strong triple bond between the two nitrogen atoms requires a large amount of energy to break, roughly equivalent to 16 ATP, so cyanobacteria only produce heterocytes under nitrogen-poor conditions (Berg et al. 2002). The micronutrient boron has been linked to the maintenance and protection of the glycolipid envelope surrounding these heterocytes, with boron deficiencies leading to the destabilization of the envelope and oxygen leakage (Downs et al. 2008, Hyenstrand et al. 2001). The availability of iron, molybdenum, and boron in freshwater systems have the potential to limit the rate of nitrogen fixation through their role as co-factors or in the maintenance of the heterocyte envelope.

The aim of my research was to investigate the effect of phosphorus, iron, boron, and molybdenum on the summer nitrogen fixation rates of 8 sites located in northern Ohio through in-lab micronutrient addition bioassays. While nitrogen fixation and the overall growth of cyanobacterial communities are frequently limited by phosphorus (Elser et al. 2007), anthropogenic activities have often led to situations in freshwater systems in which phosphorus is in excess. Based on the high energy requirement of nitrogen fixation, I hypothesized that rates of nitrogen fixation should be higher in freshwater systems that had high concentrations of phosphorus and/or low concentrations of nitrogen leading to low N:P ratios and nitrogen limitation. Furthermore, I hypothesized that iron, boron, and/or molybdenum availability would become limiting factors for cyanobacterial nitrogen fixation when phosphorus was abundant. If this study shows evidence that environmental factors limit rates of nitrogen fixation in freshwater
systems, it may further support the concept that nitrogen control can be an alternative to phosphorus control to manage algal blooms.

2.3 Methods

2.3.1 Study sites and field methods

Water samples were collected from 8 lentic sites located in northern Ohio over the summer of 2016 from July 14 – August 22. These sites included Sandy Lake, Punderson Lake, West Twin Lake, Mogadore Reservoir, Springfield Lake, Sandusky Bay, Western Lake Erie, and an unnamed lake that will be referred to as Trares Lake due to its proximity to Trares Road (Figure 2.1). The northwestern region of Ohio is relatively flat and dominated by agricultural use, particularly row crops (Ohio EPA 2008). In contrast, the northeastern region of Ohio is dominated by urban development, containing as much as 35% of the overall population of Ohio, and has a long industrial history of iron, steel, and rubber. These metropolitan areas in northeastern Ohio have aging infrastructures including outdated and leaking sewer pipes and combined sewer overflow systems. Whether impacted by agriculture in the west or urbanization in the east, northern Ohio experiences elevated levels of anthropogenic activities that can increase nutrient inputs to freshwater systems.

All water samples were collected during the early morning on sunny or partly sunny days. No collection occurred within 48 hours of a major rain event in that area. Water was collected from approximately 1 meter below the surface using 3 10-liter Nalgene bottles and immediately stored in an ice cooler. Physio-chemical measurements were recorded at the time of water collection for each of the 8 sites using a YSI 6920 V2 Sonde meter. Water samples were then transported back to the laboratory and processed within 12 hours. Precautions were taken to
avoid contamination by metals, particularly that of boron. All glassware, polycarbonate bottles, and non-metal tools that came into contact with the water samples during any time throughout the experiment were first soaked in an acid bath containing 20% hydrochloric acid, followed by four rinses with reverse osmosis (RO) purified water and three rinses with deionized water (DI). All equipment was rinsed with sample water prior to use.

2.3.2 Laboratory methods

Water samples were filtered using Whatman GF/F filter papers. Chlorophyll \(a\) samples were to be analyzed using a Fluorimeter and US E.P.A. standard method 445.0 (Arar and Collins 1997), however contamination of prepared samples from faulty equipment prevented the analysis. Concentrations of soluble reactive phosphorus and ammonium were determined using a LACHAT autoanalyzer (QuickChem FIA 8000 Series) and standard methods (Murphy and Riley 1962) which had detection limits of 9 µg SRP/L and 4 µg NH\(_4^+\)-N/L. Concentrations of nitrate
were determined using an ion chromatography (IC) machine (Dionex ICS-2100 RFIC) and standard methods (Hillman et al. 1986) which had a detection limit of 2 µg NO₃⁻-N/L.

Micronutrient addition bioassays were conducted in a laboratory no more than 12 hours after time of collection for each of the 8 sites. Polycarbonate Nalgene bottles were used for each of the 5 micronutrient bioassay treatments. The treatments were a control (received no addition of macro- or micro- nutrients), and nutrient additions were phosphorus was added singularly (P only) and in addition with iron (P + Fe), molybdenum (P + Mo), and boron (P + B). Each treatment was performed in triplicate. Additions of phosphorus were added to the micronutrient treatments to simulate nitrogen limited conditions. A solution of Na₂HPO₄ was added to increase sample concentrations of phosphorus by 1 µmol/L. Solutions of Fe(III)Cl₃ and B(OH)₃ were added to increase sample concentrations of iron and boron by 0.5 µmol/L. Lastly, a solution of Na₂MoO₄ was added to increase sample concentrations of molybdenum by 0.1 µmol/L. These concentrations were chosen based on past micronutrient addition bioassay experiments conducted in freshwater systems (Downs et al. 2008, Glass et al. 2013, Hyenstrand et al. 2001). After the nutrient additions were applied to 1 L of sample water, the 15 treatment bottles were placed under a Phillips F40T12 40-Watt growth light and allowed to incubate for 3 days at room temperature.

The rate of nitrogen fixation within each bioassay treatment was estimated using the acetylene reduction assay method. After the initial 3-day incubation time following nutrient additions, per micronutrient bioassay treatment replicate there were 4 vials that were prepared and incubated at 4 different time points covering a 24-hour period (approximately at the 3, 6, 12, 24 hours). Two sizes of sterilized glass vials were used, although the aqueous solution: gas headspace ratio was kept constant at 0.85. Each vial was filled with 17 ml (small vial) or 28 ml
(large vial) of treatment water. A syringe was used to inject each vial with 3 ml (small vial) or 6 ml (large vial) of acetylene gas. After incubation under the Phillips F40T12 40-Watt growth light, each vial was injected with 0.2 mL (small vial) or 0.3 mL (large vial) of a zinc chloride solution. Concentrations of ethylene (ppm) were determined using a syringe to withdraw 5 mL of gas from the vial headspace, which was injected into a gas chromatograph (GC-2014 Shimadzu) equipped with a hydrogen flame ionization detector. Peaks corresponding to ethylene concentrations were recorded for each vial. Using the 4 time points per bioassay treatment, the average rate of ethylene production was calculated using equations 1 and 2 that were adopted from Breitbarth et al. 2004. Equation 1 calculates the number of moles of ethylene present in the gas headspace of each vial \( n_g \), where \( P_{gT} \) is the partial pressure of ethylene (atm), \( V_g \) is the volume of the headspace (L), \( R \) is the gas constant \( (0.08206 \text{ atm L mol}^{-1} \text{ K}^{-1}) \), and \( T_o \) is the temperature at time of analysis (K).

\[
\frac{n_g = P_{gT} \cdot V_g}{R \cdot T}
\]  

Equation 2 is very similar to Eq. 1, except it calculates the number of moles of ethylene present in the aqueous solution \( n_{aq} \), where \( P_{gT} \) is the partial pressure of ethylene (atm), \( \alpha \) is the Bunsen coefficient, \( V_{aq} \) is the volume of water used (L), \( R \) is the gas constant \( (0.08206 \text{ atm L mol}^{-1} \text{ K}^{-1}) \), and \( T_o \) is the standard temperature \( (273.15 \text{ K}) \).

\[
\frac{n_{aq} = P_{gT} \cdot \alpha V_{aq}}{R \cdot T}
\]
The total number of moles of ethylene present in each vial is the sum of Eq. 1 and Eq. 2. The conversion factor of 3 moles of acetylene reduced for each mole of dinitrogen gas that is fixed was used to estimate rates of nitrogen fixation (Rice and Paul 1971).

For each site, a 45 mL sterilized plastic test tube was filled with unfiltered water and the sample was preserved by adding Lugol’s iodine solution to yield a 1% final concentration. All samples were stored at room temperature inside a closed cabinet to minimize light degradation until the phytoplankton analysis could be conducted. The Utermöhl sedimentation chamber method was used to carry out a phytoplankton enumeration analysis using standard methods (Karlson et al. 2010). The number of nitrogen-fixing cyanobacterial cells per liter for each of the 8 sites under base conditions was determined using standard methods (Lawton et al. 1999). The water from the test tube was homogenized, and a pipette was used to transfer 5 ml from each sample into a 5 ml sedimentation chamber. Due to high densities of cells, dilutions were applied when needed using deionized water (DI). Water in the sedimentation chambers were allowed to settle for approximately 24 hours, after which the sedimentation chambers were carefully placed on an inverted microscope. Each chamber was examined for nitrogen fixing cyanobacteria, with cell counts and heterocyte counts recorded for each genus of nitrogen fixing cyanobacteria that was observed. Fields of view were scanned until a total of 300–400 cells were recorded per chamber. Equation 3 was used to estimate all cell and heterocyte counts. Cell counts and heterocyte counts where then compared to the average nitrogen fixation rates of the control treatments obtained for each of the 8 sites to determine if there were any correlations.

\[
\text{Cells per ml} = \frac{\# \text{ cells} \times \text{area of chamber bottom}}{\text{Area of field of view} \times \# \text{ fields of views counted}}
\] (3)
2.3.3 Statistical Analysis

Separate ANOVA tests per site were used to test for significant differences in nitrogen fixation rates across the 5 macro- and micro-nutrient addition treatments (control, phosphorus only, phosphorus + iron, phosphorus + boron, phosphorus + molybdenum). Assumptions were checked using Levene’s test of equality of error variances, Q-Q plots, and a homogeneity test. A Tukey HSD post hoc test was used to determine which treatments were significantly different from others. Pearson’s correlation coefficient tests were used to test for relationships between nitrogen fixation rates in the control treatments for each site and variables from the phytoplankton enumeration analysis (cell counts, heterocyte counts, percent composition). Further Pearson linear correlations tested for relationships between the various additional measurements taken at each site (soluble reactive phosphorus, ammonium, and nitrate) and nitrogen fixation rates. Assumptions for linearity and homoscedasticity were checked for all Pearson linear correlation tests. All statistical analyses were performed using SPSS software. The Type I error rate was set to an alpha of 0.05 for all tests.

2.4 Results

2.4.1 Micronutrient Addition Bioassays and Chemical Analysis

Micronutrient limitation was only observed in 1 of the 8 sites, whereas macronutrient limitation was observed in 5 of the 8 sites (Figure 2.2). Rates of nitrogen fixation increased significantly in response to the addition of the macronutrient phosphorus in 5 of the 8 sites (p < 0.05). West Twin Lake had the lowest nitrogen fixation rate for the control treatment (0.004 µmol N₂/L/hr), but it had the largest increase in the nitrogen fixation rate in response to the phosphorus only treatment which increased more than 23 times the control rate (over 2,000%
increase). Mogadore Reservoir had the highest nitrogen fixation rate for the control treatment (0.178 µmol N₂/L/hr), but it had the smallest significant increase in the nitrogen fixation rate in response to the phosphorus only treatment which increased less than 2 times the control rate (under 100% increase). The further addition of the micronutrients iron and boron had no significant impact on the rates of nitrogen fixation in the 8 sites (p > 0.05). In Sandy Lake, rates of nitrogen fixation had a marginally significant increase when the micronutrient molybdenum was added along with phosphorus (p = 0.04) when compared to the single addition of phosphorus. In all remaining lakes, the further addition of molybdenum had no significant impact on the rates of nitrogen fixation in the 8 sites (p > 0.05). Sandusky Bay had the second highest nitrogen fixation rate in the control treatment (0.115 µmol L·hr⁻¹), but there was no significant difference in nitrogen fixation rates across all 5 treatments (p = 0.11) including the phosphorus only treatment. Western Lake Erie and Trares Lake both had undetectable nitrogen fixation rates across all 5 treatments.

### 2.4.2 Phytoplankton Enumeration Analysis

The cyanobacterial communities in the 8 sites varied in their density, overall richness, and percent composition. The two sites with no detectable nitrogen fixation, Lake Erie and Trares Lake, also lacked nitrogen-fixing cyanobacteria. Across the remaining 6 sites, average cell concentrations ranged from 23,499 cells/ml (West Twin Lake) to 798,6608 cells/ml (Springfield Lake). Average heterocyte concentrations ranged from 313 heterocytes /ml (West Twin Lake) to 11,615 heterocytes /ml (Springfield Lake). There was no significant correlation between nitrogen fixation rates and average cyanobacterial cell counts (r² = 0.010, d.f. = 7, p > 0.05) when all 8 sites were included. However, the removal of Springfield Lake as a possible
**Figure 2.2** N$_2$ fixation rates (mean +/- standard errors*2) in the 6 sites with detectable nitrogen fixation. Treatments included additions of phosphorus (P), P + iron (Fe), P + boron (B), and P + molybdenum (Mo). A Tukey’s HSD test was used to determine differences between treatments, with non-significantly different treatments represented by the same letter above the bars. Not shown are western Lake Erie and Trares Lake, which had undetectable N$_2$ fixation across all 5 treatments.

Outlier resulted in a significant correlation between nitrogen fixation rates and average cyanobacterial cell counts ($r^2 = 0.806$, d.f. = 7, $p = 0.006$). (Figure 2.3). There was no significant correlation between nitrogen fixation rates and average heterocyte counts ($r^2 = 0.060$, d.f. = 7, $p > 0.05$). A positive correlation was observed between average cyanobacterial cell counts and average heterocyte counts ($r^2 = 0.602$, d.f. = 7, $p = 0.02$), but became non-significant with the removal of the Springfield Lake as a possible outlier ($r^2 = 0.571$, d.f. = 6, $p > 0.05$) (Figure 2.4).

The number of nitrogen-fixing cyanobacteria genera in the 6 sites ranged from 2-4. The most abundant genera included *Cylindrospermopsis*, *Anabaenopsis*, *Aphanizomenon*, and *Dolichospermum* (Figure 2.5). Another nitrogen fixer, the genus *Anabaena*, was also observed but only in Sandusky Bay. Sandusky Bay had the largest richness of nitrogen-fixing cyanobacteria genera with a total of 4 genera. There was no significant correlation between percent composition of the 4 major nitrogen-fixing cyanobacteria genera and rates of nitrogen fixation (d.f. = 7, $p > 0.05$) (Figure 2.6).
Figure 2.3 Relationship between nitrogen fixation rates of the control treatments (μmol N₂/L/hr) and nitrogen-fixing cyanobacteria cell counts (cells/ml) for the 8 freshwater field sites with (A) and without (B) the potential outlier of Springfield Lake (SPNG). Also shown is the relationship between nitrogen fixation rates of the control treatments (μmol N₂/L/hr) and heterocyte counts (heterocytes/ml) (C).
Figure 2.4 Relationship between nitrogen-fixing cyanobacteria cell counts (cells/ml) and heterocyte counts (heterocytes/ml) for the 8 freshwater field sites with (A) and without (B) the potential outlier of Springfield Lake (SPNG).
Figure 2.5 $N_2$ fixing cyanobacteria community composition identified down to genus within each of the 6 freshwater field sites that had detectable nitrogen fixation rates: Sandy Lake (SNDY), Punderson Lake (PUND), Sandusky Bay (SBAY), West Twin Lake (WTWIN), Mogadore Reservoir (MOGA), and Springfield Lake (SPNG).
Figure 2.6 The relationship between nitrogen fixation rates of the control treatments (µmol N₂/L/hr) and the percent composition of major nitrogen-fixing cyanobacteria genera for the 8 freshwater field sites.
2.4.3 Chemical Factors

There were no significant trends between chemical measurements and rates of nitrogen fixation across the 8 sites. Soluble reactive phosphorus (SRP) concentrations were lowest in western Lake Erie and West Twin Lake (5.48 and 7.47 µg SRP/L respectively) and highest in Trares Lake (57.73 µg SRP/L). Ammonium (NH₄⁺) concentrations where lowest in Springfield Lake (2.63 µg NH₄⁺-N/L) and highest in Punderson Lake and Sandusky Bay (39.93 and 44.77 µg NH₄⁺-N/L respectively). In contrast, nitrate (NO₃⁻) concentrations were lowest in Mogadore Reservoir (2.8 µg NO₃⁻-N/L) and highest in western Lake Erie (171 µg NO₃⁻-N/L). There was no significant correlation between nitrogen fixation rates of the control treatments and soluble reactive phosphorus (r² = 0.022, d.f. = 7, p > 0.05), ammonium (r² = 0.033, d.f. = 7, p > 0.05), nor nitrate (r² = 0.102, d.f. = 7, p > 0.05). (Figure 2.7). There was also no significant correlation between the ration of ammonium: soluble reactive phosphorus and nitrogen fixation rates (r² = 0.008, d.f. = 7, p > 0.05) nor between the ratio of nitrate: soluble reactive phosphorus and nitrogen fixation rates (r² = 0.088, d.f. = 7, p > 0.05) (Figure 2.8). The concentration of all chemical compound measured for each of the 8 field sites can be found in Table 2.1.
Figure 2.7 The relationship between nitrogen fixation rates of the control treatments (µmol N₂/L/hr) and three nutrient compounds for the 8 freshwater field sites: Soluble Reactive Phosphorus (SRP), ammonium (NH₄⁺), and nitrate (NO₃⁻).
Figure 2.8 The relationship between nitrogen fixation rates of the control treatments (µmol N₂/L/hr) and two key nutrient ratios from the 8 freshwater field sites: Ammonium (NH₄⁺): Soluble Reactive Phosphorus (SRP) and nitrate (NO₃⁻): Soluble reactive phosphorus (SRP).
<table>
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<th>Site</th>
<th>Date</th>
<th>N₂ Fixation (µmol N₂/L/hr)</th>
<th>SRP (µg/L)</th>
<th>NH₄⁺ (µg/L)</th>
<th>NO₃⁻-N (µg/L)</th>
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<td>18.3</td>
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<td>15.0</td>
<td>39.9</td>
<td>12.3</td>
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<td>9.91</td>
</tr>
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<td>W. Lake Erie</td>
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<td>5.48</td>
<td>28.8</td>
<td>171</td>
</tr>
<tr>
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<tr>
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<td>2.63</td>
<td>10.3</td>
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<td>Trares Lake</td>
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<td>57.7</td>
<td>33.2</td>
<td>8.50</td>
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</table>
2.5 Discussion

2.5.1 Absence of nutrient limitation in western Lake Erie and Trares Lake

In both western Lake Erie and Trares Lake, nitrogen fixation rates were not detectable in the control, nor in any of the macro- and micro- nutrient treatments. Further analysis revealed the absence of nitrogen-fixing cyanobacteria in the phytoplankton communities at these sites. Western Lake Erie tends to be dominated by *Mycrocystis* spp. early in the summer (June-July), but the nitrogen-fixing cyanobacteria *Anabaena* spp. can become dominant or co-dominate along with *Mycrocystis* spp. later in the summer (Aug.-Sept.) when bioavailable forms of nitrogen have been depleted in the water (Chaffin et al. 2013). Algal blooms in Lake Erie are assigned a severity index determined by the biomass of the cyanobacteria, with blooms exceeding a severity level of 5 having a high risk to public health (NOAA 2016). However, the 2016 algal bloom in western Lake Erie was reported to have a severity index of only 3.2, which was much smaller than the algal bloom observed the year before which had a severity index of 10.5 (LaBarge 2017). This 2016 bloom was reported by LaBarge (2017) to be dominated by *Microcystis* throughout most of August and was concentrated near the Maumee River outlet. There was no mention of the presence or absence of *Anabaena* in the NOAA and LaBarge reports, so it may be possible that nitrogen-fixing cyanobacteria were either not present or too few in numbers to be detected. Concentrations of soluble reactive phosphorus were low for western Lake Erie compared to the other sites (5.48 µg/L), concentrations of nitrate levels were high compared to other sites (171 µg/L), and concentrations of ammonium were moderate (28.8 µg/L) compared to other sites suggesting that the N:P ratio may have been high which would have favored *Microcystis*. To my knowledge, there has been no past research on nutrient concentrations nor cyanobacterial communities in Trares Lake. Concentration of soluble reactive phosphorus was
the highest in Trares Lake (57.7 µg/L) but concentrations of reactive nitrogen were comparable to the other sites. The chemical conditions appear favorable for nitrogen fixation, but Trares Lake lacked any nitrogen fixers. This lake was instead dominated mostly by dinoflagellates.

2.5.2 Macronutrient and micronutrient limitation

Phosphorus availability was the main limiting factor on nitrogen fixation rates across the 8 sites. There was a significant increase in nitrogen fixation rates with the addition of phosphorus in 5 of the 8 sites ($p < 0.05$). A major component of RNA in ribosomes is phosphorus, which contributes a large amount to the total phosphorus requirements of cells (Elser et al. 2000). Phosphorus is also an important macronutrient required to build new cells, as the outer membrane of bacterial cells, including heterocytes, are composed of phospholipids (Flores and Herrero 2010). Furthermore, phosphorus is a component of adenosine triphosphate which is involved in the storage and transfer of energy needed to perform work (Schachtman et al 1998). The phosphorus limitation observed in 5 of the 8 sites suggests that the phosphorus requirements of the cyanobacteria are not being met despite increases in anthropogenic inputs of nutrients.

In contrast, micronutrient limitation on nitrogen fixation rates was only observed in 1 of the 8 sites. Further additions of the micronutrient molybdenum significantly increased nitrogen fixation rates when compared to the phosphorus only treatment in Sandy Lake ($p = 0.04$). Molybdenum is often present in low concentrations in aquatic systems (<20nM), which studies have shown can lead to molybdenum limitation on phytoplankton growth and nitrogen assimilation (Glass et al. 2010, Goldman 1960). Molybdenum limitation in Sandy Lake suggests that the concentrations of molybdenum are low in comparison to other required nutrients. Molybdenum limitation on nitrogen fixation rates was not observed at the other sites.
Molybdenum may have been present in higher concentrations at these sites, or despite low concentrations of molybdenum there may have been another required nutrient that was more limiting.

There was no stimulation of nitrogen fixation rates by iron or boron compared to the phosphorus only treatment across all 8 sites. The micronutrient iron, serving as a co-factor to the nitrogenase pathway that fixes nitrogen, has been shown to limit rates of nitrogen fixation and the overall growth of phytoplankton in several lake systems (Wurtsbaugh and Horne 1983, Wurtsbaugh at al. 1985). For example, in Lake Titicaca located in Peru rates of nitrogen fixation were stimulated by iron, with increases of 40-130% (Wurtsbaugh et al. 1985). Rates of nitrogen fixation were stimulated by additions of iron in Clear Lake located in California by almost 500% (Wurtsbaugh and Horne 1983). Other studies have shown that responses of phytoplankton to iron enrichment may only occur when combined with phosphorus additions (North et al. 2007). The micronutrient addition bioassay did not show a response of nitrogen fixation to iron additions, indicating that iron concentrations were high enough to meet the biological requirements of the cyanobacteria unlike Lake Titicaca and Clear Lake. The micronutrient boron, playing a role in the maintenance and stability of heterocyte envelopes, has also been shown to limit rates of nitrogen fixation through decreased nitrogenase activity (Bonilla et al. 1990, Hyenstrand et al. 2001). However, this did not appear to be the case for each of the 8 field sites. Similar to iron, the micronutrient boron may have been present in sufficient concentrations to meet the biological needs of the cyanobacteria.

2.5.3 Chemical factors vs. nitrogen fixation rates
No significant correlation was observed between nitrogen fixation rates in control treatments and concentrations of nitrogen and phosphorus at each site. As stated previously, the process of nitrogen fixation requires a large amount of energy (16 ATP) to break the double bond between the two nitrogen atoms, so phytoplankton tend to only produce the nitrogenase-containing heterocytes under nitrogen-poor conditions (Adams 2000, Berg et al. 2002). The determination of concentrations a major nitrogen and phosphorus compounds such as soluble reactive phosphorus, ammonium, and nitrate in the 8 field sites was therefore important to better interpret the results of the micronutrient addition bioassay. Because the process of nitrogen fixation costs a lot of energy, I hypothesized that rates of nitrogen fixation should be higher in sites that had high concentrations of soluble reactive phosphorus and/or low concentrations of ammonium and nitrate. However, the results of the chemical analysis did not support this hypothesis. There were no significant correlations between nitrogen fixation rates of the control treatments and soluble reactive phosphorus, ammonium, nor nitrate (P > 0.05). Nitrogen and phosphorus are key biological requirements for growth and development of phytoplankton which Redfield (1958) reported to have a specific ratio requirement of 16 nitrogen: 1 phosphorus. However, Klausmeier et al. (2004) reported that optimal phytoplankton N:P ratios can actually range between 8 and 45. It is therefore more informative to look at relative concentrations of nitrogen and phosphorus compared to nitrogen fixation rates rather than their individual concentrations. However, there was also no significant correlation between the ratio of ammonium: soluble reactive phosphorus and nitrogen fixation rates, nor between the ratio of nitrate: soluble reactive phosphorus and nitrogen fixation rates. The lack of correlation between nitrogen fixation rates and key nitrogen and phosphorus compounds may be caused by other
potential driving factors for nitrogen fixation rates in these 8 field sites, such as the phytoplankton community itself.

2.5.4 Community composition vs. nitrogen fixation rates

There was a significant positive correlation between cyanobacterial cell counts and nitrogen fixation rates when the outlier was removed (p = 0.006), but no corresponding correlation between heterocyte counts and nitrogen fixation rates (p > 0.05). Heterocytes are only produced under nitrogen-poor conditions when cyanobacteria are unable to obtain the nitrogen they need from their environment, which suggests there should be a correlation between nitrogen fixation rates and the number of heterocytes present in the cyanobacterial community (Adams 2000). The results of this study do not support this idea, which suggests that other factors might be influencing the relationship between heterocyte formation and rates of nitrogen fixation.

Biological factors might be influencing the relationship between nitrogen fixation rates and the availability of nutrients like nitrogen and phosphorus. For example, heterocytes receive nutrients and energy required for nitrogen fixation from nearby vegetative cells (Flores and Herrero 2010). Therefore, the rate of nitrogen fixation may be dependent on the number of vegetative cells that are located near the heterocyte. Furthermore, the size and composition of the cyanobacterial community can fluctuate throughout the year in response to numerous environmental factors (Bormans et al. 2005, Yannarell and Triplett 2005). For example, both temperature and light levels were able to explain some of the variation of phytoplankton community composition in canonical correspondence analysis models (Yannarell and Triplett 2005). The size of the community and overall composition have been shown to affect community characteristics. For example, cyanobacteria species both nitrogen fixers and non-nitrogen fixers
can differ in their chemical and structural defenses against grazing by zooplankton, and the phytoplankton community’s sensitivity to grazing can be influenced by the community’s species composition (Chan et al. 2004). It is therefore possible for structural and chemical defenses to determine the dominance of certain species of cyanobacteria based on grazing preferences of zooplankton, which may influence community characteristics such as nitrogen fixing capabilities. Furthermore, the efficiency of cyanobacteria to fix nitrogen can vary widely from species to species, suggesting that changes in the dominance of one species over another can lead to changes in the efficiency of the community to fix nitrogen and influence heterocysts production (Berman-Frank et al. 2007). The nitrogen-fixing cyanobacteria communities in each of the 8 field sites differed in the number of genera present and in which genera were major or minor components of the community composition. The differences in cyanobacterial community composition may influence nitrogen fixation rates, which could explain why some of the expected trends between chemical composition and nitrogen fixation rates, and between heterocyte counts and nitrogen fixation rates were not observed. However, there was no significant correlation observed between the individual percent composition of the 4 major nitrogen-fixing cyanobacteria genera and rates of nitrogen fixation ($p > 0.05$), which contrasted with the findings of Chan et al. (2004).

2.5.5 Conclusion

Nitrogen fixation rates were predominantly limited by the availability of the macronutrient phosphorus, with only a single case of micronutrient limitation observed. The high frequency of phosphorus limitation observed in the 8 field sites suggests that reductions in phosphorus inputs would be an effective management practice for these particular sites to reduce
the growth of cyanobacteria. Micronutrient limitation on nitrogen fixation rates in freshwater systems may be too rare an occurrence to aid in management techniques for the reduction of algal blooms, as supported by the fact that micronutrient limitation was only observed in 1 of the 8 sites and the significance was marginal. The lack of micronutrient limitation coupled with the frequency of phosphorus limitation supports the claims made by Schindler et al. (2016) that it is in phosphorus reductions rather than nitrogen reductions that management practices should focus on. There may still be potential for micronutrients other than iron, boron, and molybdenum to limit rates of nitrogen fixation. In conclusion, unless a frequent limitation on nitrogen fixation rates is observed, either by the availability of chemical compounds or by physical and biological factors, nitrogen reductions may prove to be an insufficient alternative to phosphorus reductions. Since not all phosphorus reduction techniques are successful at limiting the growth of cyanobacteria in the long term, it is clear that some kind of alternative control measure is required for certain freshwater systems (Lewis et al. 2011).

An important aspect of this experiment to consider is that this experiment involved small nutrient addition bioassays carried out in a laboratory setting, and the applicability of the results to the whole-ecosystem scale of the freshwater sites is unknown. Nutrient addition bioassays have been a conventional method for determining which nutrients are limiting the growth of phytoplankton (e.g. Downs et al. 2008, Goldman 1965, Hyenstrand et al. 2001). The effectiveness of using nutrient addition bioassays to determine actual limiting factors in hypereutrophic systems has been questioned, and research has tried to find alternative methods involving dilution bioassays (Paerl and Bowles 1987). These methods can be more complex, however, because a single nutrient must be removed from a water sample without altering the
concentration of other nutrients and must not have an impact from a hidden covariate on the algal community itself.

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CHAPTER 3
Applications of the acetylene reduction assay and membrane inlet mass spectrometry for measurement of nitrogen fixation

3.1 Abstract

The ability to detect low rates of nitrogen fixation in freshwater systems may differ depending on the method of analysis used. A traditional technique to measure aquatic nitrogen fixation is the acetylene reduction assay (ARA) using gas chromatography (GC). I examined a novel assay directly measuring dinitrogen gas using membrane inlet mass spectrometry (MIMS). The ARA method, although well established, has a long run time and does not directly measure dinitrogen. The MIMS assay has a much faster run time and measures nitrogen directly. However, the detection limit of the MIMS nitrogen fixation assay is unknown. To compare the detection limit of these two methods, an experiment was designed using a dilution series of a culture of the nitrogen fixing cyanobacteria *Anabaena sp.* While the ARA method was able to detect nitrogen fixation in all treatments, the assay using MIMS was only able to detect nitrogen fixation in the two treatments with the highest *Anabaena sp.* densities. Although the assay using MIMS is appealing in its quick run time and direct approach, the limit in its detectability of low nitrogen fixation rates may make it less favorable than the ARA method.

3.2 Introduction

Nitrogen fixation is a biological process that occurs in terrestrial, marine, and freshwater systems where atmospheric dinitrogen gas is converted into bioavailable ammonium by certain species of
bacteria. Accurate measurements of nitrogen fixation are important for understanding nitrogen fixation’s impact on the cycling of nitrogen. A traditional way to measure nitrogen fixation in freshwater systems is the acetylene reduction assay (ARA) (Hardy et al. 1973). The ARA method indirectly measures nitrogen fixation by first measuring the conversion rate of acetylene into ethylene and then using this information to estimate the conversion rate of dinitrogen into ammonium. Alternatively, it may be possible to measure nitrogen fixation rates directly using membrane inlet mass spectrometry (MIMS) and observing the direct drawdown of dinitrogen gas through time Kana et al. 1994). Here I examined whether a novel assay using MIMS can be developed as an alternative to the ARA method. The goal was to offer an assay with the ability to measure changes in dissolved dinitrogen gas more quickly and directly than the ARA method.

ARA is a traditional method for measuring nitrogen fixation, however one disadvantage of this method is that it measures ethylene, not nitrogen, and requires a conversion factor to indirectly determine nitrogen fixation. Dilworth (1966) observed that the nitrogen-fixing enzyme nitrogenase also reduces acetylene gas into ethylene gas, which suggested that acetylene reduction could be used to estimate nitrogen fixation rates. A benefit of the ARA method is its simplistic procedure and relatively short incubation time (Knowles and Blackburn 1993). In the ARA method, acetylene gas is injected into water samples and, after the incubation period (0-24 hours), gas from the headspace is analyzed for ethylene using gas chromatograph. The rate of ethylene production is often directly reported, or it is converted into a rate of nitrogen fixation using a conversion factor. The typical conversion used is 1 mole of dinitrogen gas fixed for every 3 moles of ethylene gas produced. A benefit of the ARA method is that concentrations of ethylene in the hemisphere are extremely low (150 ppt), which results in minimal atmospheric ethylene contamination of the water samples (Aikin et al. 1982). Lower atmospheric
contamination of a measured compound makes it easier to observe small changes in the concentration of that compound, such as with ethylene in water samples. However, prolonged exposure of bacteria to acetylene has been shown to negatively affect the physiology of the microorganisms (David and Fay 1977). Furthermore, accurate conversion between rates of ethylene production into rates of dinitrogen breakdown requires the ARA method to be calibrated using $^{15}$N-N$_2$ isotope methods, and the actual conversion coefficient can range from 2 – 56 moles of ethylene produced for every mole of dinitrogen that is fixed (Belnap 2001). These issues can lead to an inaccurate representation of the natural nitrogen fixation rates in the freshwater system.

The $^{15}$N-N$_2$ stable isotope technique mentioned above is another traditional method for measuring nitrogen fixation. Labelled $^{15}$N-N$_2$ is introduced to the sample, which is then fixed by cyanobacteria and incorporated into organic tissue (Neess et al. 1962). The amount of labelled N in the organic tissue can then be determined and used to estimate rates of nitrogen fixation. A benefit of the $^{15}$N-N$_2$ stable isotope method is the direct measurement of nitrogen fixation through the monitoring of $^{15}$N labelled N$_2$ that is converted into $^{15}$N labelled organic nitrogen inside the cells of cyanobacteria. Disadvantages of the $^{15}$N-N$_2$ stable isotope method include long incubation times and greater expenses compared to other methods (Knowles and Blackburn 1993). Furthermore, the sensitivity of this method to detect nitrogen fixation is $10^3$-fold less than the ARA method (Hardy et al. 1968). Until the novel MIMS nitrogen fixation assay can be further validated, it did not seem warranted to compare the assay using MIMS to the more expensive isotopic method. Therefore, my method comparison relied on the less expensive and more sensitive ARA method.
Membrane inlet mass spectroscopy has existed for some time, however to my knowledge the technology has not been applied to the task of quantifying nitrogen fixation. MIMS was originally developed in 1963 as a way to measure the kinetics of oxygen during photosynthesis (Hoch and Kok 1963). Since then, the applicability of MIMS has expanded to include analysis of gases in air, gas exchange rates across soils to monitor microbial processes, and dissolved gases associated with fermentation (Ketola and Lauritsen 2016, Lloyd et al. 2002). The use of MIMS to look at gases dissolved in marine and freshwater systems is a relatively new application compared to the ARA method (Schlüter and Gentz 2008). The assay using MIMS operates by drawing up a continuous stream of sample water, which passes through a semipermeable silicon rubber tube under vacuum. The silicon tubing acts as a semipermeable membrane, allowing dissolved gases such as nitrogen, oxygen and argon to diffuse out of the water and enter a liquid nitrogen cooled trap (Kana et al. 1994). Water vapor and carbon dioxide are removed by the trap, and the remaining gases enter the mass spectrometer for analysis based on molecular mass. A benefit of developing an assay using MIMS is that it can directly measure the drawdown of dinitrogen gas in a water sample through time as it is being removed by the process of nitrogen fixation. However, the ability of the assay using MIMS to detect low levels of nitrogen fixation is unknown. Unlike the case with ethylene, a sizable portion of the atmosphere is N₂ gas (78% by volume), which diffuses between the atmosphere and freshwater systems. With the large background pool of N₂, it may be difficult to adequately detect small changes in the concentration of N₂ that occur during nitrogen fixation.

To investigate the ability of the assay using MIMS to detect low levels of nitrogen fixation measurements were compared to the ARA method using a culture of the nitrogen-fixing cyanobacteria to create treatments with a gradient of cell densities and nitrogen fixation rates. I
predicted that the ARA method would be able to detect low nitrogen fixation rates in treatments associated with lower concentrations of *Anabaena sp.* due to its ability to detect small changes in the concentration of ethylene gas. In contrast, I predicted that the assay using MIMS would only be able to detect nitrogen fixation rates in treatments that contain the higher concentrations of *Anabaena sp.* since it will be more difficult to detect a small change in the large pool of dinitrogen gas. Along the gradient of *Anabaena sp.* concentration treatments from high concentration to low concentration of *Anabaena sp.*, I predict there will be a point in which the assay using the MIMS can no longer detect nitrogen fixation whereas the ARA method will continue to detect nitrogen fixation. Results in support of these predictions would indicate that an assay using MIMS for determining nitrogen fixation may not be an alternative to the ARA method unless the rates of nitrogen fixation were expected to be high such as during a bloom event.

3.3 Methods

3.3.1 *Anabaena sp.* cultures

A monoculture of living *Anabaena sp.* item #151710 was obtained from Carolina Biological Supply in 2015 and was placed in a large glass container with filtered water from Sandy Lake and incubated under a Phillips F40T12 40-Watt growth light for a year. A COMBO growth medium was created using standard methods (Kilham et al. 1998). The medium included CaCl$_2$, MgSO$_4$, K$_2$HPO$_4$, NaNO$_3$, NaHCO$_3$, Na$_2$SiO$_3$, H$_3$BO$_3$, and KCl. Also included in the COMBO medium were trace elements including FeCl$_3$, MnCl$_2$, CuSO$_4$, ZnSO$_4$, CoCl$_2$, NaMoO$_4$, H$_2$SeO$_3$, and Na$_3$VO$_4$. Small amounts of this growth medium were added to the *Anabaena sp.*
culture periodically to promote growth, until approximately three weeks prior to the start of the experiment.

The *Anabaena sp.* culture was examined under a microscope every couple of days until heterocysts became visible indicating the availability of NO₃⁻ supplied by the growth medium had been depleted and the system had entered nitrogen-poor conditions. A series of dilution treatments were established using filtered water from Sandy Lake, the *Anabaena sp.* culture, and 1 L Nalgene bottles. The treatments were a control (0 ml culture : 600 ml lake water), 0.03 dilution factor (15 ml culture : 585 ml lake water), 0.05 dilution factor (30 ml culture : 570 ml lake water), 0.10 dilution factor (60 ml culture : 540 ml lake water), 0.20 dilution factor (120 ml culture : 480 ml lake water), 0.40 dilution factor (240 ml culture : 360 ml lake water) and 0.80 dilution factor (480 ml culture : 120 ml lake water). The treatments created a gradient of *Anabaena sp.* concentrations to represent a gradient of nitrogen fixation rates (Figure 3.1).

![Figure 3.1](image)

**Figure 3.1** The gradient of *Anabaena sp.* cultures used as treatments for the method comparison analysis between the acetylene reduction assay method (ARA) and the membrane inlet mass spectrometry method (MIMS). Shown from left to right is the control followed by 6 treatments that range from low to high concentrations of *Anabaena sp.* cells.
For each treatment bottle, 12 ml of the culture: lake water mixture was used to fill 4 MIMS vials such that no air bubbles remained in the vial once the cap was added. This was repeated 3 times per treatment. All MIMS vials were incubated under a Phillips F40T12 40-Watt growth light, with the 4 vials per treatment incubated for different lengths of time to span a 24-hour period. After the designated incubation period, each vial was injected with 0.2 mL of a zinc chloride solution to arrest biological activity. These vials were stored at room temperature until they could be analyzed on the MIMS. The concentration of N₂ (ppm) in each vial was determined using standard methods for MIMS analysis (Kana et al. 1994). From the 4 time points per treatment, the average rate of N₂ drawdown and overall nitrogen fixation was calculated.

For comparison, the ARA method was also used to estimate rates of nitrogen fixation within each treatment. For each treatment bottle, 17 ml of the culture: lake water mixture was used to fill 4 ARA vials, with a headspace volume of 20 ml. This was repeated 3 times per treatment. A syringe was used to inject 3 ml of acetylene gas into each vile. All ARA vials were placed under the same Phillips F40T12 40-Watt growth light as the MIMS vials, with the 4 vials per treatment incubated for different lengths of time to span a 24-hour period. After the designated incubation period, each vial was injected with 0.2 mL of a zinc chloride solution. For each vial, a sterile syringe was used to withdraw 5 mL of gas from the vial headspace, which was injected into a gas chromatograph (GC-2014 Shimadzu) equipped with a hydrogen flame detector. Peaks corresponding to ethylene were recorded and using the 4 time points per treatment the average rate of ethylene production was calculated using equations 1 and 2 adopted from Breitbarth et al. 2004. Equation 1 calculates the number of moles of ethylene present in the gas headspace of each vial (n₉), where P₉ is the partial pressure of ethylene (atm), V₉ is the
volume of the headspace (L), R is the gas constant (0.08206 atm L mol⁻¹ K⁻¹), and T₀ is the temperature at time of analysis (K).

\[ n_g = \frac{P_gT \cdot V_g}{R \cdot T} \]  

Equation 2 is very similar to Eq. 1, except it calculates the number of moles of ethylene present in the aqueous solution (n_{aq}), where P_{gT} is the partial pressure of ethylene (atm), α is the Bunsen coefficient, V_{aq} is the volume of water used (L), R is the gas constant (0.08206 atm L mol⁻¹ K⁻¹), and T₀ is the standard temperature (273.15 K).

\[ n_{aq} = \frac{P_{gT} \cdot \alpha \cdot V_{aq}}{R \cdot T} \]  

The total number of moles of ethylene present in each vial is the sum of Eq. 1 and Eq. 2. The conversion factor of 3 moles of acetylene reduced for each mole of dinitrogen gas that is fixed was used to estimate rates of nitrogen fixation (Rice and Paul 1971). These values were then compared to the rates of nitrogen fixation obtained from the assay using MIMS.

### 3.3.2 Statistical analysis

Linear regression tests were used to determine the relationship between nitrogen fixation rates and time of incubation for the *Anabaena sp.* treatments, using both the ARA method and the assay using MIMS. Values that were significantly different from zero were determined to be detectable rates of nitrogen fixation. Another linear regression test was used to test for a relationship between nitrogen fixation rates and the dilution factor of the *Anabaena sp.* cultures for the ARA method. A similar test could not be performed for the assay using MIMS due to the
small number of treatments with detectable nitrogen fixation rates. Assumptions for linearity and homoscedasticity were checked for all linear regression tests. All statistical analyses were performed using SPSS software, and the Type I error rate was set to an alpha of 0.05 for all tests.

3.4 Results

The ARA method had a greater ability to detect low nitrogen fixation rates compared to the assay using MIMS, and the actual value of nitrogen fixation within a treatment differed between methods. The treatments of *Anabaena sp.* cultures analyzed using the ARA method ranged in their rates of nitrogen fixation from 0.001 µmol N₂/L/hr for the 0.03 dilution factor treatment to 0.033 µmol N₂/L/hr for the 0.80 dilution factor treatment (Figure 3.2). The only treatment that had undetectable nitrogen fixation rates when analyzed with the ARA method was the control treatment, which received no additions of the nitrogen-fixing cyanobacteria *Anabaena sp.* ($r^2 = 0.064$, d.f. = 11, $p = 0.43$). In contrast, the treatments of *Anabaena sp.* cultures analyzed using the assay MIMS had no detectable nitrogen fixation rates in multiple treatments ranging from the control to the 0.20 dilution factor treatment. Only in the two highest treatments, the 0.40 dilution factor treatment and the 0.80 dilution factor treatment, was there a statistically significant nitrogen fixation rate ($r^2 = 0.612$ and 0.494 respectively, d.f. = 11, $p < 0.05$). The rates of nitrogen fixation of the 0.40 dilution factor treatment and the 0.80 dilution factor treatment when analyzed with the assay MIMS 0.886 µmol N₂/L/hr and 1.02 µmol N₂/L/hr respectively. There was a significant, positive relationship between the dilution factors used in the treatments of *Anabaena sp.* cultures and the rates of nitrogen fixation for the ARA method ($r^2 = 0.966$, d.f. = 6, $p < 0.001$). A linear regression test was not used to test for a relationship between the dilution factors used in the treatments of *Anabaena sp.* cultures and the rates of
Figure 3.2 The relationship between nitrogen fixation rates (µmol N₂/L/hr) and the dilution factor of the *Anabaena sp.* culture that was added to filtered pond water, compared across the two methods: (A) acetylene reduction assay (ARA) and (B) membrane inlet mass spectrometry (MIMS). Rates listed as zero were non-detectable and are marked with a *.
nitrogen fixation for the assay using MIMS because only 2 of the 7 treatments had detectable nitrogen fixation rates.

3.5 Discussion

The differences in the ability to detect low nitrogen fixation rates for the ARA method and the assay using MIMS offers support that the ARA method may be a more practical method for research of nitrogen fixation rates in aquatic systems. The significant, positive relationship between nitrogen fixation rates and the dilution factor of the *Anabaena sp.* culture for the ARA method shown in figure 3.2 indicates that the experimental design was successful at creating treatments that included a gradient of *Anabaena sp.* culture solutions. The ARA method, which was able to detect nitrogen fixation rates across all treatments except the control, was able to detect a range of 0.001 µmol N₂/L/hr to 0.033 µmol N₂/L/hr. This range of detectable nitrogen fixation rates for the ARA method offers an effective way to measure low levels of nitrogen fixation in freshwater systems during non-bloom time periods. For example, Lake George was reported to have nitrogen fixation rates that ranged from 0.003 µmol N₂/L/hr to as high as 0.050 µmol N₂/L/hr which the ARA method would have been able to fully detect (Horne and Viner 1971). However, the nitrogen fixation rates on the lower end of this range would have been beyond the capabilities of the assay using MIMS.

The assay using MIMS was not as well suited to detect low levels of nitrogen fixation as the ARA method. The assay using MIMS could not detect rates of nitrogen fixation below the 0.40 dilution factor treatment. The fact that the lower dilution factor treatments were non-detectable for the assay using MIMS suggesting that this method may not be an effective way to measure low levels of nitrogen fixation in freshwater systems unless there were a large bloom.
event. For example, a large bloom event that occurred in Lake Windemere was reported to have nitrogen fixation rates that were above 1.0 μmol N₂/L/hr and would have been able to be detected by the assay using MIMS (Horne and Fogg 1970). However, the assay using MIMS would not have been able to detect all of the nitrogen fixation rates reported for Lake George mentioned above (Horne and Viner 1971). However, even if the assay using MIMS was able to detect nitrogen fixation in these two lakes discussed here, the values obtained would be questionable. Irregularities were observed between the nitrogen fixation rates calculated using the ARA method and those calculated by the assay using MIMS. The two highest dilution factor treatments measured with the assay using MIMS had nitrogen fixation rates of 0.886 μmol N₂/L/hr and 1.02 μmol N₂/L/hr respectively. The nitrogen fixation rates for the two highest dilution factor treatments were higher than those estimated using the ARA method, which were 0.020 μmol N₂/L/hr and 0.033 respectively.

Possible explanations for the difference in rates of nitrogen fixation between the two methods include using the incorrect conversion factor, issues with air bubbles in the water samples, and machine error. The experimentally determined conversion factor of nitrogen-fixing cyanobacteria is 3 moles of ethylene produced for every mole of nitrogen fixed, which is the most common conversion factor reported (Hardy et al. 1973). This conversion factor is not precise and has been reported to range from 2 – 56 moles of ethylene produced for every mole of dinitrogen that is fixed, most likely due to a lack of calibration of the ARA method using ¹⁵N-N₂ isotope methods (Belnap 2001). The nitrogen fixation rates observed with the assay using MIMS were much high than those observed using the ARA method, indicating that a lower conversion factor may be needed. However, using a conversion factor of 1-2 instead of 3 would not drastically change the values of nitrogen fixation rates observed for the ARA method. The
typically accepted conversion factors are not able to account for the differences between rates determined by the ARA method and the assay using MIMS. Another weakness of using MIMS in general is that water samples must be free of bubbles, as bubbles can alter the concentration of gases dissolved in water. Typically, bubbles will reduce the concentration of gases dissolved in the sample as they instead diffuse into the bubble. The treatments that had observable nitrogen fixation rates when analyzed with the assay using MIMS were the treatments with the two highest cell densities for *Anabaena sp*. When there are more cyanobacteria present, the rates of photosynthesis and oxygen production will be greater. If enough oxygen is released into the water sample, bubbles may start to form which can strip nitrogen from the water through diffusion. While bubbles were not noted in the samples, it is possible the samples were not examined in close enough detail. If bubbles were present in the samples, this might explain the extra drawdown of nitrogen gas through time, with the nitrogen removal assumed to be from nitrogen fixation when in reality the nitrogen diffused into the bubbles. It is also possible that there may have been calibration issues with the equipment used to analyze the samples.

In conclusion, there are multiple ways of detecting nitrogen fixation in freshwater systems, but differences in the ability of the methods to detect low levels of nitrogen fixation should be considered. The assay using MIMS is appealing due to its simplicity of procedure and because it measures nitrogen directly instead of estimating nitrogen fixation from ethylene production. However, the ARA method appears to be better capable of detecting nitrogen fixation when the abundance of nitrogen fixers is low, compared to the method using MIMS. Unless sampling is occurring during a high-density event such as a harmful algal bloom, there is no clear indication that the MIMS method will be useful for detection of nitrogen fixation. Even then, the high rates of nitrogen fixation calculated with the assay using MIMS that could not be
well explained suggests that problems with the assay using MIMS may be more complicated than just a detection limit issue.

References


Table A.1 Cell counts and heterocyte counts for nitrogen-fixing cyanobacteria across the 8 field sites are listed, along with their corresponding nitrogen fixation rates for control treatments.

<table>
<thead>
<tr>
<th>Site</th>
<th>Date</th>
<th>N₂ Fixation (µmol N₂/L/hr)</th>
<th>Cyanobacterial Cell Count (cells/ml)</th>
<th>Heterocyte Count (Heterocytes/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandy Lake</td>
<td>07/14/2016</td>
<td>0.017</td>
<td>5,5500</td>
<td>8,850</td>
</tr>
<tr>
<td>Punderson Lake</td>
<td>07/22/2016</td>
<td>0.012</td>
<td>27,500</td>
<td>1,010</td>
</tr>
<tr>
<td>Sandusky Bay</td>
<td>07/27/2016</td>
<td>0.115</td>
<td>60,500</td>
<td>2,140</td>
</tr>
<tr>
<td>Western Lake Erie</td>
<td>08/03/2016</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>West Twin Lake</td>
<td>08/04/2016</td>
<td>0.004</td>
<td>23,500</td>
<td>313</td>
</tr>
<tr>
<td>Mogadore Reservoir</td>
<td>08/11/2016</td>
<td>0.178</td>
<td>107,000</td>
<td>5,280</td>
</tr>
<tr>
<td>Springfield Lake</td>
<td>08/18/2016</td>
<td>0.043</td>
<td>799,000</td>
<td>11,600</td>
</tr>
<tr>
<td>Trares Lake</td>
<td>08/22/2016</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table A2 Summary of field measurements for various physio-chemical properties for each of the 8 freshwater systems in the micronutrient addition bioassay experiment.

<table>
<thead>
<tr>
<th>Site</th>
<th>Date</th>
<th>Temperature (°C)</th>
<th>Conductivity (µs/cm)</th>
<th>D.O. (mg/L)</th>
<th>% D.O</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandy Lake</td>
<td>07/14/2016</td>
<td>26.97</td>
<td>551</td>
<td>10.98</td>
<td>134.7</td>
<td>8.8</td>
</tr>
<tr>
<td>Punderson Lake</td>
<td>07/22/2016</td>
<td>26.5</td>
<td>505</td>
<td>8.67</td>
<td>107.6</td>
<td>8.68</td>
</tr>
<tr>
<td>Sandusky Bay</td>
<td>07/27/2016</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>W. Lake Erie</td>
<td>08/03/2016</td>
<td>27.77</td>
<td>284</td>
<td>9.63</td>
<td>121.2</td>
<td>9.52</td>
</tr>
<tr>
<td>W. Twin Lake</td>
<td>08/04/2016</td>
<td>28.49</td>
<td>606</td>
<td>9.49</td>
<td>123</td>
<td>8.87</td>
</tr>
<tr>
<td>Mogadore Reservoir</td>
<td>08/11/2016</td>
<td>26.92</td>
<td>407</td>
<td>9.07</td>
<td>116</td>
<td>8.95</td>
</tr>
<tr>
<td>Springfield Lake</td>
<td>08/18/2016</td>
<td>25.6</td>
<td>774</td>
<td>10.71</td>
<td>132.9</td>
<td>8.83</td>
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<tr>
<td>Treares Road Lake</td>
<td>08/22/2016</td>
<td>23.42</td>
<td>573</td>
<td>10.27</td>
<td>126.3</td>
<td>9.24</td>
</tr>
</tbody>
</table>

*Note: Some values for Sandusky Bay were missing from the collected data.